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NEUROMUSCULAR BLOCKING IN ACUTELY TETANUS INTOXICATED MICE

NAKABA SUGIMOTO and MORIHIRO MATSUDA

Department of Tuberculosis Research I, Research Institute for Microbial Diseases, Osaka University, Yamadaoka, Suita, Osaka 565, Japan

YOSHITO OHNUKI, TERUO NAKAYAMA and KYOKO IMAI

Department of Physiology, Osaka University Medical School, Kita-ku, Osaka 530, Japan

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SUMMARY The effects of tetanus toxin on neuromuscular transmission of mice in acute intoxication produced by intravenous injection of a large amount of the toxin were examined by (1) recording the phrenic nerve impulses, the electromyograms (EMGs) of the diaphragm and the electrocardiograms, and (2) the evoked EMGs of the gastrocnemius muscle in response to electrical stimulation of the sciatic nerve. The evoked EMGs of the gastrocnemius muscle were analyzed in terms of kinetic and tonic components by their different latencies. Just before death of animals, the EMGs of the diaphragm appeared with some delay relative to the corresponding phrenic discharges. Finally, the EMG of the diaphragm disappeared even in the presence of phrenic discharge, but cardiac electrical activities continued. The amplitudes of the evoked EMGs of the gastrocnemius muscle invariably became low before death, but the muscle action potential could be recorded by direct muscle stimulation for several minutes after death. The latencies of the evoked EMGs were constant until about the middle of the survival time when the latencies suddenly became prolonged. The longer latency was the same as that of the tonic action potentials. Thus, in acutely tetanus-intoxicated mice, neuromuscular transmission was blocked rapidly and the kinetic component of the muscle was blocked earlier than the tonic component.

INTRODUCTION

Tetanus toxin has been reported to act on the central nervous system, mainly causing hyperactivity of the motor system followed, after a latent period of several hours or days, by signs of spastic paralysis, which is the main symptom of typical tetanus in man and animals.

Besides central effects, tetanus toxin has been reported to show peripheral effects. Flaccid paralysis was observed in some clinical cases of severe tetanus (Kaeser et al., 1968). Local flaccid paralysis was also observed under particular experimental conditions. When rabbits were administered with antitoxin in the

early stage of tetanus intoxication (Miyasaki et al., 1967) and when mice were pretreated with a large amount of toxoid (Davis and Wright, 1955), they showed paralytic signs, instead of spastic signs, with intramuscular injection of tetanus toxin. When the toxin was injected into the anterior chamber of the eye, pupillary paralysis was produced (King et al., 1978). Blocking effects of tetanus toxin on neuromuscular transmission have been demonstrated experimentally in local tetanus induced by intramuscular injection of the toxin into mice (Duchen and Tonge, 1973), cats (Takano, 1976), rats (Kretzschmar et al., 1980) and goldfish (Mellanby and Thompson, 1981). Habermann et al. (1980) demonstrated that the tetanus toxin molecule itself blocks neuromuscular transmission in vitro in a mouse phrenic nerve-hemidiaphragm preparation. However, in whole animals, the participation of the peripheral action in tetanus intoxication is unclear.

We found the intravenous injection of purified tetanus toxin (1000–0.06 μg) killed mice within 20–450 min after signs of flaccid paralysis (Matsuda et al., 1982). Since the signs were indistinguishable from those produced by botulinum toxin, which blocks neuromuscular transmission, we examined neuromuscular transmission in acute tetanus intoxication in mice electrophysiologically.

The evoked EMG recorded with a surface electrode, has been proved to be a vector summation of two components, kinetic and tonic activities (Nakayama and Hori, 1967a). When a nerve is stimulated repetitively at a rate of more than 25 per sec, the tonic component is abolished and a " pure " kinetic response is obtained in the evoked EMG of the corresponding muscle (Nakayama and Hori, 1970). The latency of the kinetic component is shorter than that of the tonic component, because the conduction velocity of the kinetic motor fiber is faster than that of the tonic motor fiber (Eccles et al., 1958; Nakayama and Hori, 1967b). Thus, analysis of the evoked EMG is a simple method for studying nerve-muscle conduction.

This paper reports EMG studies on phrenic nerve-diaphragm and sciatic nerve-gastrocnemius muscle preparations in acutely tetanus-intoxicated mice.

MATERIALS AND METHODS

Experiments were performed on 91 OFl mice weighing 18 to 31 g. Tetanus toxin was prepared and purified from an extract of *Clostridium tetani* Harvard A47, substrain Biken, by the method of Matsuda and Yoneda (1975). The purified preparation of toxin contained 365–390 flocculating units/mg of protein and 0.8 to 1.6×10^7 minimum lethal doses/mg of protein. The preparation was homogeneous, forming a single precipitation band against crude horse antitoxin in immunodiffusion plates and migrating as a single protein band on polyacrylamide gel electrophoresis. In experiments, 0.1–0.2 ml of toxin solution (6–12 mg/ml in 0.1 *M* KNa phosphate buffer, pH 7.5) was injected into a tail vein of mice.

For recording of the phrenic impulses and EMG of the diaphragm, mice were anesthetized with sodium pentobarbital, 50 μg/g i.p., and were fixed in the supine position. A phrenic nerve on the right side of the neck was isolated from surrounding tissues under a dissecting microscope and an implantable electrode was fixed on it. The EMG of the diaphragm was recorded with a bipolar needle electrode inserted into the diaphragm through a small hole in the right upper abdominal wall. The phrenic nerve was identified by the nerve activities synchronized with the EMG of the diaphragm. The activities of the phrenic nerve and the EMG of the diaphragm were monitored continuously with an oscilloscope and loud speaker and simultaneously recorded on magnetic tapes with an FM magnetic data recorder.

For recording the evoked EMG of the gastrocnemius muscle, the right sciatic nerve was isolated from surrounding tissues at the level of the upper thigh. An implantable electrode for stimulation was fixed to the nerve. The gastrocnemius muscle was exposed for inserting a bipolar needle electrode. The sciatic nerve was stimulated every 10 sec by single rectangular pulses (duration, 0.01–0.1 msec; intensity, 1.3–6.8 V). The evoked EMGs of the gastrocnemius muscle were amplified and monitored

on an oscilloscope, photographed directly and led into a memory oscilloscope for later reading out.

Throughout the studies, all the electrodes were covered with a mixture of vaseline and paraffin oil to prevent their drying and the influences of exudate.

RESULTS

1. *Effects of acute tetanus intoxication on the activities of the phrenic nerve and diaphragm*

Figure 1 (A-F) shows the electrical activities of the phrenic nerve and diaphragm just before and after death of an intoxicated mouse. In this case, the respiratory frequency (RF) was 100/min and the heart rate (HR) was 406/min before toxin injection (Fig. 1A). In the early period of 0–90 min after the injection, the RF and HR showed almost linear decreases in rate of 0.8/min and 1.7/min, respectively. In the period of 90–147 min after the injection, RF was 26 ± 2/min and HR was 250 ± 4/min. From about 143 min after the injection, the phrenic nerve activities were occasionally not accompanied by EMG activities of the diaphragm, and irregular respiration became evident. At about 147 min, the RF and HR suddenly decreased to 6/min and 24/min, respectively (Fig. 1B). Just before death, at about 148 min 30 sec (Fig. 1C), the EMG of the diaphragm began to show remarkable delay relative to the corresponding phrenic burst. When the delay reached 700 msec, respiratory movement ceased. Figure 1D shows a burst of phrenic discharges and sporadic activities of the diaphragm EMG independent of the phrenic burst discharges. Figure 1E shows the continuous discharges of the diaphragm but no phrenic discharge. The continuous discharges of the diaphragm lasted for about one minute. At 151 min 10 sec, neither the phrenic impulse nor the EMG of the diaphragm were observed while the electrocardiogram was still recorded (Fig. 1F).

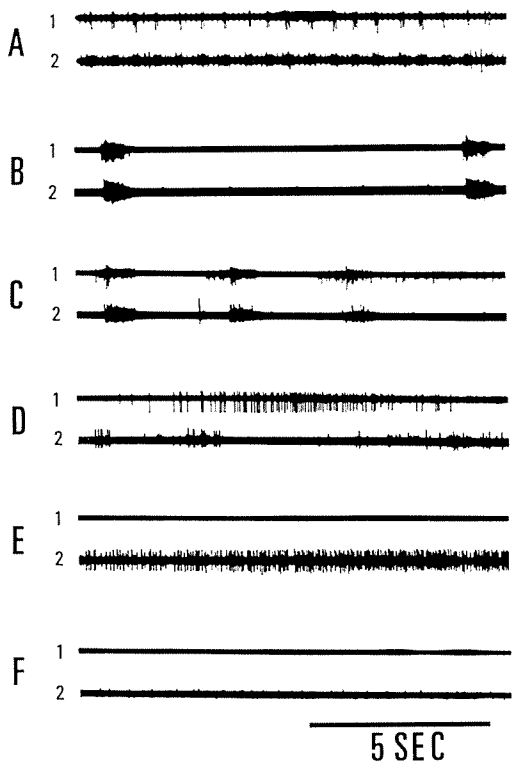

FIGURE 1. Recording of impulses from the phrenic nerve and EMG of the diaphragm during acute tetanus intoxication. A mouse weighing 37 g was anesthetized with 1.8 mg sodium pentobarbital and was injected with 2.4 mg purified tetanus toxin. The mouse died 151 min after the injection. During the course of intoxication, the impulses from the phrenic nerve (1) and the EMG from the diaphragm (2) were recorded. Electrocardiograms were seen as small deflections in trace 2.
A, just before toxin injection; B, 147 min; C, 148 min 30 sec; D, 149 min; E, 150 min 20 sec; F, 151 min 10 sec (shortly after death), after toxin injection.

2. *EMG of the gastrocnemius muscle evoked by electrical stimulation of the sciatic nerve in intoxicated mice*

The evoked EMGs of the gastrocnemius muscle were recorded every 10 sec during intoxication and some of them are presented in Fig. 2A. Figure 2B shows the change of latencies, which are the times from stimulation

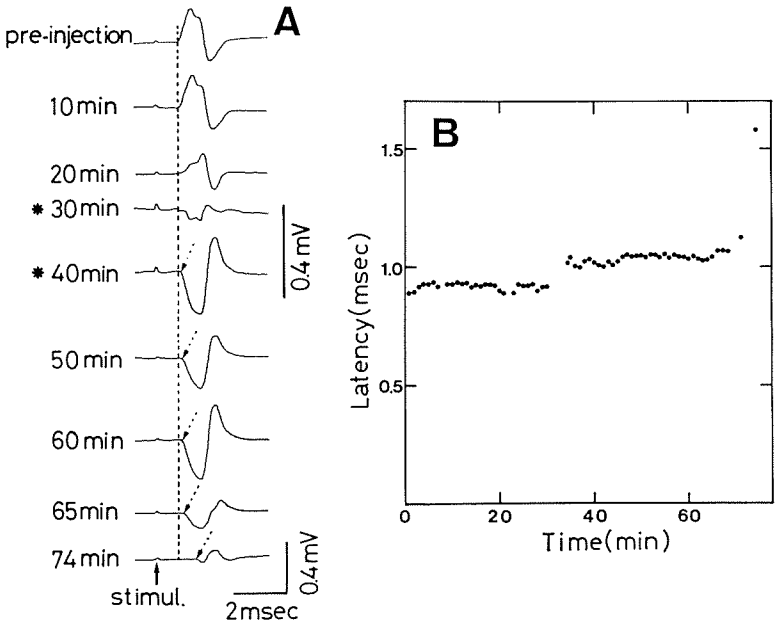

FIGURE 2. Evoked EMGs of the gastrocnemius muscle and their latencies during acute tetanus intoxication. A mouse weighing 18 g was injected with 840 μg purified tetanus toxin and died 75 min later. The sciatic nerve was stimulated every 10 sec by rectangular pulses (duration, 0.1 msec; intensity, 0.1 V). A, series of evoked EMGs recorded at the indicated times after the injection. Dotted arrows indicate the start of evoked EMGs with longer latency. At 30 min(*) and 40 min(*) after the injection, the evoked EMGs were recorded at higher magnification as indicated by the calibration. B, latencies of evoked EMGs plotted against the time after the injection. At about 30 min after the injection, the evoked EMGs became too small to measure their exact latencies.

to the onset of the evoked EMGs. The latencies of the evoked EMGs were unchanged from 0.92 ± 0.02 msec until 30 min after toxin injection. After 35 min, the latencies were 1.03 ± 0.02 msec. The longer latency lasted until about 10 min before death (64 min after the injection). Latencies increased abruptly to 1.65 msec at death (Fig. 2B).

The peak-to-peak amplitudes of the evoked EMGs became very low in the middle of intoxication and then gradually became high until about 15 min before death in this case. But the evoked EMG is a vector summation of the kinetic and tonic activities, and so the peak-to-peak amplitude of the compound muscle action potential is not always proportional to the mechanical response of the muscle. The peak-to-peak amplitudes of the evoked EMGs invariably became very low in the late stage of intoxication. Soon after death when an EMG could no longer be evoked by stimulation of the nerve, the muscle could contract in response to direct muscle stimulation.

3. *Analysis of the kinetic and tonic components of the evoked EMG of the gastrocnemius muscle before and after intoxication*

To analyze the effects of intoxication on the evoked EMG more precisely, we first tried to find out if the evoked EMGs of the gastro-

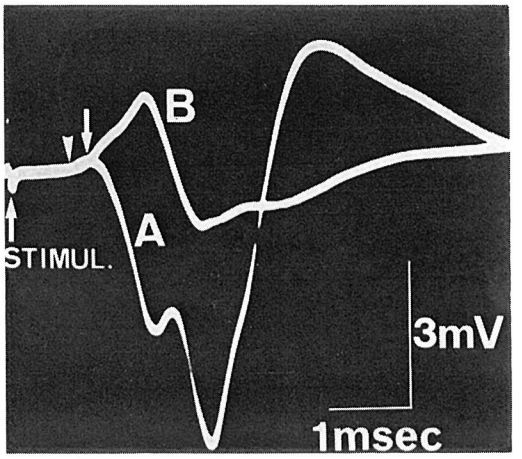

FIGURE 3. Component analysis of evoked EMG of the gastrocnemius muscle before intoxication. Wave A was obtained by a single stimulation (duration, 0.01 msec; intensity, 1.3 V). Wave B was obtained 18 sec after the beginning of repetitive stimulation at 50 Hz for 30 sec. The two waves are superimposed in the figure. The short arrow indicates the onsets of waves A and B. The long arrow indicates the point at which wave A and B separate.

cnemius muscle of the mouse were also composed of two components; i.e., kinetic and tonic components.

Wave A in Fig. 3 is the action potential evoked by a single rectangular stimulus (duration, 0.01 msec; intensity, 1.3 V). The wave had a latency of 0.67 msec, showing first an upward peak at 0.94 msec, then downward deflection with a notch at 1.74 msec and then upward deflection. Wave B in Fig. 3 was obtained 18 sec after the beginning of repetitive stimulation at 50 Hz for 30 sec. The wave B was considered to be composed of only the kinetic component because the tonic component is abolished by repetitive stimulation, as mentioned above. In Fig. 3, the wave B has the same latency of 0.67 msec as that of the wave A. Trace of the wave B is the same as that of the wave A initially, but is separated from the trace of the wave A at 0.94 msec, as shown by a long arrow (Fig. 3). As the wave A in Fig. 3 is a vector summation of kinetic

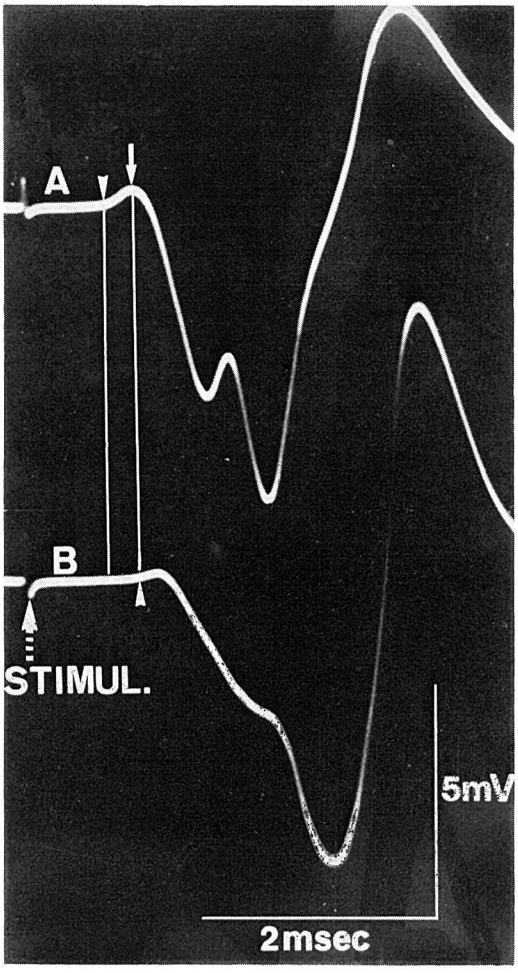

FIGURE 4. Evoked EMGs with shorter and longer latency obtained in the early and late stages of intoxication. The mouse weighing 32 g used in the experiment for Fig. 3 was injected with 1.2 mg tetanus toxin and died 117 min later. The sciatic nerve was stimulated every 10 sec by single rectangular pulses under the same conditions as for wave A in Fig. 3. All the evoked EMGs were photographed directly on the oscilloscope. Wave A obtained 4 min after the injection had a short latency. Wave B obtained 90 min after the injection had a longer latency. The short arrows indicate the onset of each wave. The long arrow indicates the onset of the tonic component of wave A.

and tonic activities, the point at which waves A and B are separated indicates the onset of

tonic activity. Thus, the latency of the tonic component in this case was estimated to be 0.94 msec. The conduction velocities of motor fibers can be calculated from latencies of the evoked EMG and distances between stimulating and recording electrodes. The mouse is too small and its sciatic nerve is too short to measure the exact length between the stimulating and recording electrodes. However, the ratio of the conduction velocities of the tonic and kinetic motor fibers could be calculated as a ratio of the latencies between the kinetic and tonic components, i.e., 0.67 msec/0.94 msec=0.71. This value was in good agreement with those of Eccles et al. (1958) and Nakayama and Hori (1967b).

The series of evoked EMGs during intoxication was recorded. The evoked EMGs could be divided into two groups by their latencies as in the experiment for Fig. 2. The evoked EMG with the shorter latency and that with the longer latency are shown in the Fig. 4. Wave A in Fig. 4, recorded at 4 min after toxin injection, had the same characteristics as wave A in Fig. 3: latency, 0.67 msec; first upward peak, 0.94 msec; notch, 1.74 msec. Therefore, wave A in Fig. 4 was considered to be composed of the two components, kinetic and tonic. Wave B in Fig. 4, obtained at 90 min after the injection, had a latency of 0.94 msec. This coincided with the latency of the tonic component calculated in the experiment for Fig. 3. Therefore, wave B in Fig. 4 was concluded to be composed mainly of the tonic component.

DISCUSSION

Our results indicated that block of neuromuscular transmission occurred in acutely tetanus-intoxicated mice that showed signs of apparently generalized flaccid paralysis. The gastrocnemius muscle responded to direct muscle stimulation after death and phrenic nerve impulses were observed at the final stage of intoxication. Thus, the lack of a muscle response observed in acute tetanus intoxication was due to block of neuromuscular transmission.

Results also showed that the effect of tetanus toxin in blocking neuromuscular transmission occurred within minutes in our preparation. Although most studies on the peripheral effects of tetanus toxin have been performed on local tetanus several hours or days after toxin injection (e.g., Duchen and Tonge, 1973; Takano, 1976; King et al., 1978; Kretzschmar et al., 1980; Mellanby and Thompson, 1981), our results indicated that tetanus toxin did not require a long latent period for eliciting peripheral effects. In our system, the intoxication was so rapid that we could observe the peripheral effects of the toxin without complication from central effects, since the central effects require a long incubation period (Habermann and Erdmann, 1978). Thus, mice acutely intoxicated with tetanus toxin provide a simple model for studying the primary effect of the peripheral action of the toxin.

Furthermore, the results clearly demonstrated that there were two definite stages in the peripheral effects of tetanus toxin. In the middle of intoxication, a period of shorter latency changed to that of longer latency in the response of the gastrocnemius muscle to sciatic stimulation (Fig. 2). The earlier shorter and later longer latencies coincided with those of the kinetic and tonic components, respectively, of the evoked EMG (Fig. 3, 4). Therefore, there were two stages in acute tetanus intoxication: an early stage in which both the kinetic and tonic components responded to nerve stimulation and a later stage in which the fast kinetic component could no longer respond and only the slow tonic component responded to nerve stimulation. Finally, neuromuscular transmission of the tonic component was also blocked and the mice died. In acute tetanus intoxication, the fast white kinetic muscle component was blocked more readily than the slow red tonic muscle component.

In local tetanus of mice (Duchen and Tonge,

1973), cats (Takano, 1976) and rats (Kretzschmar et al., 1980) by intramuscular injection of tetanus toxin, different sensitivities to the toxin of neuromuscular transmission in the two types of muscles, fast and slow, have been observed. Duchen and Tonge (1973) reported strong sensitivity of the soleus muscle of mouse to tetanus toxin, and concluded that slow muscles are more sensitive than fast muscles to the peripheral action of tetanus toxin. On the contrary, Takano (1976) reported that neuromuscular transmission in the fast muscle was more sensitive to the toxin than that in the slow muscle in cats. Kretzschmar et al. (1980) demonstrated that the percentage of white fibers in the muscles, estimated histologically, displays a very good negative correlation with the minimum dose of toxin needed for total blocking of neuromuscular transmission in rats. The results of our EMG studies on gastrocnemius muscle of mice in acute tetanus intoxication were in good agreement with those of Takano (1976) on cats and Kretzschmar et al. (1980) on rats.

In addition to blocking neuromuscular transmission, tetanus toxin have systemic effects in acute intoxication, since both the respiratory frequency and the heart rate decreased in the early stage of the intoxication, and these changes cannot be explained simply by block of neuromuscular transmission.

The survival time of intoxicated mice in these experiments was longer than that reported by Matsuda et al. (1982). Since in this study, animals were treated with anesthetics before toxin injection, anesthetics might prolong the survival time. But the anesthetics used did not affect neuromuscular transmission.

By component analysis of evoked EMGs on acute intoxication, we could observe the peripheral effect of tetanus toxin from beginning to death and found differentiating action of the toxin on two components of muscle. Analysis of the components of the evoked EMG will be useful in understanding the mechanisms of action of various end-plate-blocking agents such as botulinum toxin. Tetanus toxin, which has a differentiating action on fast and slow components of muscle, may be useful in neuropharamacological studies.

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